Does coenzyme-Q have a protective effect against atorvastatin induced myopathy? A histopathological and immunohistochemical study in albino rats

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Summary. Introduction. In addition to their lipid-lowering effect, statins have pleiotropic effects that may extend their use to the treatment and prevention of various other diseases such as cancer, osteoporosis, multiple sclerosis, rheumatoid arthritis, type 2 diabetes, and Alzheimer’s disease. Consequently, the number of patients taking statins is expected to increase. A side effect of statins, statin-induced myopathy, which may result from reduced muscular coenzyme Q10 levels, limits their use. The current study investigates if supplementing with CoQ10 could ameliorate statin induced myopathy. Materials and Methods. Forty adult male albino rats were randomized into 4 groups, with 10 rats per group. The following was administered to the rats using oral gavage for 4 weeks: Group 1: 2 ml of 0.5% carboxymethyl cellulose once daily. Group 2: 100 mg/kg/ day coenzyme Q10 dissolved in 2 ml of cotton seed oil. Group 3: 10 mg/kg once daily atorvastatin dissolved in 0.5% carboxymethyl cellulose. Group 4: concomitantly received CoQ10 and atorvastatin similar to groups 2 and 3 respectively. Plasma creatine kinase levels were measured by using spectrophotometer. The right extensor digitorum longus muscle sections were stained for histological (Haematoxylin & Eosin, Masson trichrome and Phosphotungstic acid haematoxylin) and immunohistochemical (cytochrome C and Bax) examinations. Quantitative measures of cytochrome C and Bax were carried out using image analyzer.

Results. Atorvastatin induced increased total creatine kinase, skeletal muscle variations in the sizes and shapes, necrosis, disorganization, nuclear pyknosis, karyorrhexis, karyolysis, dismantled plasma membrane, excess collagen fibers and lipid deposition in addition to loss of cross striation. Atorvastatin increased the intensity of the immune-positive reactions of cytochrome C and Bax. These changes were ameliorated by concomitantly giving coenzyme Q10.

Conclusion: CoQ10 may ameliorate atorvastatin induced skeletal muscle injury.

Key words: Atorvastatin, CoQ10, Skeletal muscle, Histological, Immunohistochemical

Introduction

Statins are the most commonly used drugs for reducing hypercholesterolemia (Marcoff and Thompson, 2007). Rhabdomyolysis induced acute renal failure has been reported as a very serious but rare adverse effect of statins (Ucar et al., 2000). What may more commonly limit their use is the occurrence of myopathy, in the form of fatigue, muscle pain, muscle tenderness, muscle weakness and nocturnal cramping. Increased serum creatine kinase (CK) levels may or may not be associated with statin-induced myopathy. Myopathy is more likely to occur with lipophilic statins which are commonly used, such as atorvastatin, simvastatin and lovastatin compared to the hydrophilic statins such as pravastatin, rosuvastatin, and fluvastatin (Kiener et al., 2001).

The myotoxic effect of lipophilic statins, including
atorvastatin, is attributed to their penetration to the muscle tissue. Statins primarily affect the mitochondria of the skeletal muscle by decreasing the synthesis of mevalonate, which is a critical intermediary in the cholesterol synthesis pathway, via the competitive inhibition of HMG-CoA reductase. The inhibitory effect of statins on HMG-CoA reductase leads to a reduction of the farnesyl pyrophosphate synthesis which is an intermediate in the synthesis of CoQ10 (ubiquinone) (Marcoff and Thompson, 2007).

Coenzyme Q10 (CoQ10), a fat-soluble, vitamin-like benzoquinone compound, is also known as ubiquinone-10 or ubiquinol-10. CoQ10 is located in the mitochondria, lysosomes, Golgi and plasma membranes (Sohal et al., 2007). CoQ10 is essential for the production of cellular energy and its antioxidant properties. The latter occurs by the direct reaction of CoQ10 with free radicals and regeneration of tocopherol and ascorbate from their oxidized state (Sohal et al., 2007). Cellular energy production occurs through its role in adenosine triphosphate (ATP) synthesis in the mitochondrial inner membrane and in stabilization of the cell membranes (Päivä et al., 2005). Low serum coenzyme Q10 could induce impaired mitochondrial enzyme activity (Sohal et al., 2007).

These facts have supported the idea that statin induced myopathy could be attributed to decreased CoQ10 in muscular tissue (Marcoff and Thompson, 2007).

In addition to their cholesterol lowering effect, statins have cholesterol-independent pleiotropic effects that may extend their use to the treatment and prevention of other diseases such as cancer, osteoporosis, multiple sclerosis, rheumatoid arthritis, Type 2 diabetes mellitus, and Alzheimer’s disease (Carmena and Betteridge, 2004; Liao and Laufs, 2005; Sleijfer et al., 2005). Consequently, the number of patients taking statin therapy is expected to increase and it becomes essential to attempt to decrease the occurrence of statin-induced myopathy.

The current study investigates whether supplementing with CoQ10 could prevent or ameliorate the myotoxic effects of atorvastatin in albino rats.

Materials and methods

A total of 40 adult male albino rats (200–250 g) were maintained with water and food ad libitum at constant humidity and temperature. The animals were randomized into 4 groups (10 animals in each group).

Group 1 (control 1): rats received 2 ml of 0.5% carboxymethyl cellulose through oral gavage.

Group 2 (control 2): rats orally (by oral gavage) received CoQ10 (Sigma Chemical Company, St. Louis, MO, USA) in a dose of 100mg/kg/ day dissolved in 2ml of cotton seed oil (Özdoğan et al., 2012).

Group 3 (atorvastatin treated group): rats orally (by oral gavage) received 10 mg/kg/ day atorvastatin (Pfizer, Inc., New York, NY) (Pierno et al., 2006; Schmechel et al., 2009) dissolved in 0.5% carboxymethyl cellulose (Tsujihata et al., 2008).

Group 4 (atorvastatin and CoQ10 treated group): rats concomitantly received CoQ10 and atorvastatin similar to groups II and III respectively.

Four week later, the animals were anaesthetized by ether inhalation before exsanguinations. Blood samples were collected from cardiac puncture. The extensor digitorum longus muscle of the right leg was removed and prepared for histological and immunohistochemical studies.

The animal manipulations were performed in the Laboratory Animal Center of College of Medicine, King Saud University (Riyadh, KSA) in accordance with the institutional and national guide for the care and use of laboratory animals. The experiment was approved by The Ethical committee (Institutional Review Board), of Prince Sultan Bin Abdulaziz College for EMS, King Saud University.

The following investigations were conducted using the collected samples and specimens:

Biochemical analysis

The plasma creatine kinase (CK) activity levels were measured by standard spectrophotometric analysis by using diagnostic kit (Sigma Aldrich). Plasma level of creatine kinase (CK) activity is considered as the biomarker for the diagnosis of myopathy and muscle breakdown (Pierno et al., 2006).

Histological and immunohistochemical studies

The extensor digitorum longus muscle (Pierno et al., 2006) of each rat was dissected and fixed in 10% neutral buffered formalin solution (Sigma Chemical Co., St. Louis, MO) for 24 h. The muscle specimens were processed to prepare 4 µm-thick paraffin sections. The sections were stained for histological and immunohistochemical studies.

Histological studies

Haematoxylin and Eosin (H&E) (Drury and Wallington, 1980) Masson trichrome (Bancroft et al., 1994) and Phosphotungstic acid haematoxylin (PTAH) (Avwioro, 2010) were used to verify histological details and the presence of collagen and cross striation respectively in the muscle’s sections.

Immunohistochemical studies

Paraffin sections were mounted on positively charged slides and processed for Bar (B-9, Santa Cruz, USA) and Cytochrome C (A-8, Santa Cruz, USA) immunohistotochemistry. After deparaffinization in xylene, immunohistochemistry was performed using a 3-
step indirect process based on the labelled avidin biotin peroxidase complex (ABC) method. Sections were rehydrated in descending grades of alcohol. Following blocking of endogenous peroxidase activity with 3% H2O2 in methanol and non-specific binding sites with a protein blocker, the sections were incubated for 32 minutes with a 1:100 dilution of Bax and Cytochrome C primary antibody (Rabbit anti-rats Bax and Cytochrome C polyclonal antibody, Santa Cruz, USA). Then, biotinylated secondary antibody was added at a concentration of 2% for 30 minutes (37ºC) followed by addition of the avidin-biotin peroxidase complex (ABC). Visualization of the reaction was performed using 3, 3-diaminobenzidine (DAB) as the chromogen which produces a dark brown precipitate that is readily detected by light microscopy. The sections were then counterstained with Mayer’s hematoxylin, dehydrated in ascending grades of alcohol, cleared in xylene and mounted with DPX (the Bax and Cytochrome C cytoplasmic and membranes sites of reaction were stained brown and nuclei were stained blue/brown). The negative control included sections which were incubated in the absence of the primary antibody.

The sections were independently examined by the histologist and pathologist authors (KMS & KN).

Quantitative analysis

Quantitative measurements were carried out using image analyzer (Super eye-Heidi soft, Histology Department, Faculty of Medicine, King Saud University, Saudi Arabia). Ten random high-power fields (Objective, x400) in each slide were selected and captured for each group. The optical density (OD) of Bax and cytochrome C brown color reactions of the cytoplasm of the muscle was measured. The color area percent of the blue color (collagen) in Masson’s Trichrome stained sections was also measured. The image analyzer was calibrated for color measurement before using.

Statistical analysis

The data were analyzed using SPSS statistical software version 19. The means of color area percent and OD of the immune-positive immunohistochemical reactions among the studied groups were compared by using ANOVA-test. The significance level was considered at p value ≤0.05.

Results

Biochemical analysis

The means (U/L±SEM) levels of CK were significantly higher (ANOVA <0.001) in group 3 (189±9) compared to group 1 (130±8), group 2 (137±6), and group 4 (154±3).

Macroscopic findings

No changes were detected grossly in the muscles of group 1 (Control 1), group 2 (Control 2), group 3 (atorvastatin treated group) and group 4 (CoQ10 and atorvastatin treated group).

Microscopic findings

Groups 1 and 2 (Control Groups)

In control groups the H&E histological staining of these groups showed normal muscle structure (Fig. 1A-D) and normal sarcoplasmic cross striations detected by PTAH stain (Fig. 1E).

The immunohistochemical staining of Bax showed that some muscle fibers were moderately immunopositive (Fig. 1F). The Cytochrome C showed faint immunopositive reaction detected at the periphery of few muscle fibers while the rest revealed immunonegative reaction (Fig. 1G).

Group 3 (atorvastatin treated group)

The H&E stained LS revealed disorganized, fragmented and discontinued muscle fibers. The nuclei revealed pyknotic, karyorrheic and karyolytic changes. Some nuclei were internal rather than peripheral to the muscle fibers (Fig. 2A). In the transverse sections, some fibers showed necrosis, hypereosinophilic staining and were surrounded by a halo of clear external rims reflecting increased lipid deposition, atrophic, circular rather than polygonal and had internal nuclei (Fig. 2B).

The Masson Trichrome stained sections revealed excess collagen fibers around the affected areas. The affected muscle fibers showed infiltration with lipocytes (Fig. 2C). Some muscle fibers stained blue with bright red irregular subsarcolemmal depositions (Fig. 2D).

The PTAH showed loss of cross striation of the affected fibers (Fig. 2E)

The immunohistochemical studies of the Bax and Cytochrome C showed marked immunopositive staining of the majority of muscle fibers (Fig. 2F,G).

Group 4 (atorvastatin and CoQ10 treated group):

The H&E stained LS (Fig. 3A) and TS (Fig. 3B) examination revealed intact, polygonal muscle fibers with peripherally located nuclei nearly similar to the controls. The Masson Trichrome stained sections revealed that a minimal amount of collagen separated the muscle fibers (Fig. 3C,D).

The PTAH stained sections revealed focal loss of cross striation of sarcoplasm of few muscle fibers (Fig. 3E) however the majority of muscle fibers were normal.

The immunohistochemical studies of Bax showed some of the muscle fibers were moderately immunopositive stained (Fig. 3F).
Cytochrome C revealed mild immunopositive reaction in some muscle fibers while it was only markedly detected in few fibers. The rest of the fibers revealed immune-negative reaction (Fig. 3G).

Quantitative analysis of histological and immunohistochemical parameters

The mean of color area percent of Masson Trichrome stain in the atorvastatin treated group (Group 3) was significantly higher than the other groups (p value <0.0001). The optical density (OD) of Bax and Cytochrome C immunopositive expressions were also significantly higher in the same group compared to the others (p value for ANOVA <0.0001) (Table 1).

Discussion

The extensor digitorum longus muscle was chosen for this study because it is mainly formed of type II

Table 1. The quantitative histological and immunohistochemical analysis of the different groups.

<table>
<thead>
<tr>
<th></th>
<th>Group 1</th>
<th>Group 2</th>
<th>Group 3</th>
<th>Group 4</th>
<th>ANOVA</th>
</tr>
</thead>
<tbody>
<tr>
<td>Masson trichrome</td>
<td>0.032±0.006</td>
<td>0.035±0.003</td>
<td>0.146±0.006*</td>
<td>0.039±0.004</td>
<td>0.0001</td>
</tr>
<tr>
<td>Bax (Mean OD±SD)</td>
<td>0.0029±0.0006</td>
<td>0.0034±0.0004</td>
<td>0.399±0.032*</td>
<td>0.0166±0.009</td>
<td>0.00001</td>
</tr>
<tr>
<td>Cytochrome C (Mean OD±SD)</td>
<td>0.103±0.06</td>
<td>0.088±0.02</td>
<td>0.352±0.18*</td>
<td>0.118±0.075</td>
<td>0.0001</td>
</tr>
</tbody>
</table>

*Statistically significant comparing to other groups

Fig. 1. Control extensor digitorum longus muscle. A. Muscle nuclei (arrow); fibroblast (arrow head) (LS section; H&E). B. Muscle nuclei (arrow); fibroblast (arrow head) (TS section; H&E). C. Collagen (arrow) (LS section; Masson Trichrome). D. Collagen (arrow) (TS section; Masson Trichrome). E. Cross striations of muscle fibers (arrows) (LS section PTAH). F. Immunopositive Bax staining (arrows) (TS section; DAB&H). G. Immunopositive Cytochrome C staining (arrows) (TS section; DAB&H).
white fibers (Soukup et al., 2002) which are selectively subjected to statin induced myotoxicity (Nakahara et al., 1988; Westwood et al., 2005).

In the current study, atorvastatin induced a significant increase in total serum CK, which indicates muscle injury (Omar et al., 2001; Füzi et al., 2012). This is in accordance with the findings of Füzi et al. and Dodiya and colleagues who reported that statins induced muscle injury, demonstrated by decreased muscle force and elevated serum, CK levels in rats (Füzi et al., 2012; Dodiya et al., 2013).

Histologically, the current study showed that atorvastatin induced increased eosinophilic staining, variation in sizes and shapes, and necrosis, in addition to disorganization of muscle fibers. Atorvastatin treated rats also showed dismantled plasma membrane of the muscle fibers, internal located nuclei, and nuclear muscle fiber pyknosis, karyorrhexis and karyolysis. This finding is in accordance with other studies which showed that the dismantled plasma membrane and pyknosis reflect the presence of apoptosis induced by atorvastatins (Sacher et al., 2005; Savill and Fadok, 2000). Moreover, in this study, atorvastatin also induced excess collagen, lipid deposition, and loss of cross striation of the muscle fibers.

The current study detected some muscle fibers which were surrounded by clear external rims (halos), reflecting increased lipid deposition around these fibers (Sacher et al., 2005). The Masson Trichrome of the current study also detected some muscle fibers stained blue with bright red irregular subsarcolemmal depositions and accumulated lipids within the myocytes. These changes are characteristic of mitochondrial disorder (Lammens and Laak, 2004).

The accumulated lipids within the myocytes could be attributed to the fact that atorvastatin induces dysfunctional mitochondrial chain respiration; a metabolic abnormality which could predispose to the accumulation of lipids within myocytes and consequently causes myopathy (Mastaglia, 1982;

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Fig. 2. Atorvastatin treated group. A. Internal nuclei (arrow); Disorganized and fragmented muscle fibers (arrow head); pyknosis (P_k); karyorrhexis (Kh); karyolysis (K) (LS section; H&E). B. Internal nuclei (arrow); degenerated myofibrils (arrow head); hypereosinophilic atrophic muscle fibers (star) surrounded by a halo of clear external rims (circle); Circular muscle fibers (◊); Dismantled muscle fibers (D) (TS section; H&E). C. Collagen surrounding the muscle fibers (arrow); Collagen within the muscle fibers (arrow head); Lipocyte (star) (LS section; Masson Trichrome). D. Blue stained muscle fibers with bright red irregular subsarcolemmal depositions (arrows) (TS section; Masson Trichrome) E. Striations of the muscle fibers (arrow); No striation (arrow head) (LS section PTAH). F. Immunopositive Bax staining (arrows) (TS section; DAB&H). G. Immunopositive Cytochrome C staining (arrows); Immunonegative staining (star) (TS section; DAB&H).
Phillips et al., 2002). The accumulated lipids can occur with or without increased creatine kinase levels (Phillips et al., 2002).

Atorvastatin inhibits the mevalonate formation which is a precursor of cholesterol produced by 3-hydroxy-3-methylglutaryl coenzyme A (HMG-CoA) reductase. Mevalonate is a precursor also of ubiquinone (coenzyme Q10) which has an antioxidant and membrane stabilizer effect. COQ10 is utilized by mitochondria for electron transport (Bliznakov, 2002; Deichmann et al., 2010). By inhibiting the production of ubiquinone, statins may alter membrane properties and inhibit the production of mitochondrial adenosine triphosphate (ATP) and consequently impair energy metabolism of myocytes (Chu et al., 1997; Bliznakov, 2002). There is a growing body of evidence indicating that statins may induce myopathic changes via a decrease in ubiquinone levels (Watts et al., 1993; Bargossi et al., 1994; Bliznakov and Wilkins, 1998; Schaefer et al., 2004) which can be reversed by COQ10 supplements (Bargossi et al., 1994). Moreover, statins induce mitochondrial skeletal muscle reactive oxygen species (ROS) production which triggers deleterious effects on mitochondrial function (Boutbir et al., 2012).

Immunohistochemically, the current study showed increased immunopositive staining of cytochrome C and Bax in the atorvastatin treated group. The atorvastatin induced rupture and loss of outer mitochondrial membrane which leads to permeabilization of cytochrome C from the mitochondrial intermembrane space to the cytoplasm (Kaufmann et al., 2006). Cytoplasmic cytochrome C can activate caspases which induce apoptosis (Zamzami and Kroemer, 2003).

The deleterious effects of atorvastatin on skeletal muscle are most probably due to apoptosis which involves the mitochondrial pathway. This involves the release of cytochrome C into the cytosol (Adhihetty and Hood, 2003) as a sequence of the increased permeability of mitochondrial transition pore for Bax and/or mitochondrial production of ROS (Boutbir et al., 2012).

Fig. 3. Atorvastatin and COQ10 treated group. A. (LS section; H&E). B. (TS section; H&E). C. (LS section; Masson Trichrome). D. (TS section; Masson Trichrome). E. Striations of the muscle fibers (arrow); No striation (arrow head) (LS section PTAH). F. Immunopositive Bax staining (arrows) (TS section; DAB&H). G. Mild (arrows), Moderate (arrow head), Negative (star) Cytochrome C immuno staining; (TS section; DAB&H).
The ROS can directly induce the dissociation of cytochrome C from the inner mitochondrial membrane and cause its subsequent release into cytosol (Nomura et al., 2000). The ROS can also indirectly trigger the apoptotic pathway via activating mitogen activated protein kinases (MAPKs) and various other redox-sensitive transcription factors which are involved in both anti- and proapoptotic gene expression (Bogoyevitch et al., 2000; Hood, 2001). The ROS activates the MAPKs by targeting its thiol group. This alters the conformation of the MAPKs and increases its activity (Bogoyevitch et al., 2000; Hood, 2001).

Bax may also redistribute to the endoplasmic reticulum, resulting in depletion of stored calcium which results in caspase activation (Zong et al., 2003; Oakes et al., 2005), which may also result in apoptosis.

The current study showed that CoQ10 ameliorated atorvastatin induced skeletal muscle histological and immunohistochemical changes. The atorvastatin+CoQ10 (Group 4) treated rats maintained intact, polygonal and cross striated muscle fibers, with minimal deposition of collagen and peripheral located nuclei. The immunohistochemical investigations showed that CoQ10 decreased cytochrome C and Bax immunopositive expression in Group 4 compared to Group 3.

The data in the literature regarding the association between the administration of statins and the myocellular levels of CoQ10 are inconsistent. Laaksonen and his colleagues showed that statins induce decreased levels (Laaksonen et al., 1995); however others showed no changes (Lamperti et al., 2005) of myocellular levels of coenzyme Q10. Furthermore, the decreased myocellular CoQ10 concentration did not cause histochemical or biochemical evidence of mitochondrial myopathy or morphological evidence of apoptosis in most patients (Lamperti et al., 2005). Conversely, another study by Muraki and colleagues showed that coenzyme Q10 treatment reversed atorvastatin induced mitochondrial dysfunction, decreased oxygen utilization, and thus improved exercise tolerance (Muraki et al., 2012).

The beneficial effect of CoQ10 which was found in the current study could be attributed to its support of adenosine triphosphate (ATP) synthesis in the mitochondrial inner membrane and by stabilizing the cellular and mitochondrial membranes, thus preserving cellular integrity and function. CoQ10 is also a potent antioxidant that blocks oxidative injuries to DNA, lipids, proteins, and other essential molecules (Palomaki et al., 1998).

The current study has some limitations in that it did not include a physiological study of the rat muscle and also did not detect muscular COQ10 in the studied groups. The authors recommend that future studies on the supplementation of COQ10 on statin treated animals should be conducted to reach evidence-based conclusions on the exact mechanism of its beneficial effect and the value of its concomitant use by patients receiving statins.

In conclusion, the current study has shown that atorvastatin causes skeletal muscle damage in rats which could be minimized by concomitantly administering coenzyme Q10.

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Author's contribution
M.S. Khalil: provided the idea, methodology, commented on the pathological changes, data analysis, and writing the draft
N. Khamis: developed the methodology, commented on the pathological changes, reviewed and finalized the paper
A.M. Al-Drees: developed the statistics, commented on the immunohistochemical changes and finalized the paper
H.M. Abdullghan: developed the methodology, supervised the drug administration in animal house, performed statistical studies, and finalized the paper.

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